

RAPID detection and quantification of *E. coli* O157/O26/O111 in minced beef by real-time PCR

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ABSTRACT

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Aim: To develop a real-time PCR detection procedure for *Escherichia coli* O111, O26 and O157 from minced meat.

Methods and Results: Strains ($n = 8$) of each of *E. coli* O26, *E. coli* O111 and *E. coli* O157 were inoculated at *ca* 10–20 CFU g⁻¹ into minced retail meat and enriched for 6 h at 41.5°C as follows: *E. coli* O26 in tryptone soya broth (TSB) supplemented with cefixime (50 µg l⁻¹), vancomycin (40 mg l⁻¹) and potassium tellurite (2.5 mg l⁻¹); *E. coli* O111 in TSB supplemented with cefixime (50 µg l⁻¹) and vancomycin (40 mg l⁻¹); *E. coli* O157 in *E. coli* broth supplemented with novobiocin (20 mg l⁻¹). DNA was extracted from the enriched cultures, and detected and quantified by real-time PCR using verotoxin (*vt1* and *vt2*) and serogroup (O157 *per gene*; O26 *fliC-fliA* genes and O111 *mzy* gene) specific primers.

Conclusions: The methods outlined were found to be sensitive and specific for the routine detection of *E. coli* O111, O26 and O157 in minced beef.

Significance and Impact of the Study: The enrichment, isolation and detection procedures used in this study provide a rapid routine-based molecular method for the detection and differentiation of *E. coli* O26, O111 and O157 from minced meat.

Keywords: *Escherichia coli* O111, *Escherichia coli* O157, *Escherichia coli* O26, real-time PCR.

INTRODUCTION

Verocytotoxigenic *Escherichia coli* (VTEC) are highly virulent food poisoning pathogens, causing a range of symptoms from mild to bloody diarrhoea, haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura, leading in some cases to permanent disability or death (Karmali 1989).

Such illness although initially associated with the consumption of contaminated undercooked ground beef (Doyle 1991), is now linked with consumption of a wider range of food products including vegetables, fish, milk and cheese (De Boer and Heuvelink 2001). Although *E. coli* O157 is currently the most widely recognized VTEC, having been implicated in cases of human infection from over 30

countries in six continents (Tozzi *et al.* 2001), other serogroups including O26 and O111 are being increasingly associated with human diseases (Johnson *et al.* 1996). These non-O157 VTEC strains are heterogeneous in their phenotypic properties and as a result, few laboratories screen for them in clinical or food samples (Johnson *et al.* 1996).

Published cultural methods for the isolation and identification of *E. coli* O157, O111 and O26 from contaminated food are time consuming and labour intensive (ISO 2001; Catarame *et al.* 2003). This situation causes particular difficulties in relation to routine food testing, especially in circumstances where customers and statutory authorities require 'evidence of the absence' of the pathogen. Therefore, there is an increasing need in the food industry to access rapid and sensitive detection methods.

Molecular detection methods based on PCR are increasingly accepted as alternatives to conventional cultural/biochemical methods for the detection of bacterial

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contamination in food (De Boer and Beumer 1999). Conventional PCR assays require amplification of a target gene in a thermocycler, separation of PCR products by gel electrophoresis, followed by visualization and analysis of the resultant electrophoretic patterns. However, this process can take a number of hours. More recently, real-time PCR is being increasingly used as a rapid sensitive and specific molecular diagnostic technique for the testing and identification of food pathogens from biological and environmental samples (Bellin *et al.* 2001). The advantages of real-time PCR technology includes speed (*ca* 30–40 min per 40 PCR cycles) and the ability to continuously monitor the progress of the PCR in each sample after each cycle. Although expensive in capital terms, real-time PCR-based strategies are becoming more popular in research and public health laboratories and are being increasingly used for specific diagnostic applications and pathogen detection (Bellin *et al.* 2001; Taylor *et al.* 2001). However, the overall potential of real-time PCR in the food industry has, in the past, been limited by the general pattern of low target cell numbers in suspect foods, and the compounds which interfere with or inhibit the progress of the PCR.

A number of suitable gene targets have been suggested for the detection and/or differentiation of VTEC. These include the *rfb* region of the bacterial genome which codes for immunodominant surface molecules on the O-side chain of bacterial lipopolysaccharides. This *rfb* region varies in length between 8 and 14 kb, and contains eight to 14 genes encoding the enzymes necessary for the synthesis of the O side chains that confer serogroup specificity. Closely related strains of *E. coli* can have different *rfb* genes encoding different O antigens (Liu and Matsumura 1994). Other genes targeted by recently developed protocols include verotoxin genes (Paton and Paton 1998; Bellin *et al.* 2001; Sharma 2002), and *E. coli* O157 and O111 species-specific genes (portions of the *rfb* O-antigen encoding regions) (Paton and Paton 1998). Although a real-time PCR method has been developed for the detection of nonserotype-specific VTEC species carrying the major VTEC associated genes, i.e. *eae*, *vt1* and *vt2* of O157, O111 and O26 (Sharma 2002), to date there is no real-time PCR system for the specific detection and or differentiation of clinically significant serogroups such as O26, O157 and O111.

This study aimed to develop a real-time PCR method for the detection and quantification of VTEC (*E. coli* O157, O26 and O111) from inoculated minced beef using the Light-Cycler System (Roche, Mannheim, Germany), including:

- i Development of a real-time PCR procedure for the detection of verotoxin genes (*vt1* and *vt2*) from *E. coli* O157, O26 and O111.
- ii Development of a real-time PCR protocol for the detection of *E. coli* O157 and O111 species-specific genes.

- iii Identification of a gene or gene region of *E. coli* O26 which could be used in the species-specific identification of this serogroup, and development of a real-time PCR procedure for the identification of this organism from contaminated minced beef.

The study also aimed to examine the relative amounts of VTEC DNA and total bacterial DNA recovered by a range of suitable DNA extraction methods, to develop more sensitive protocols for the detection of VTEC DNA from typical complex food matrices.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli serogroup O26 isolates ($n = 8$) and *E. coli* serogroup O111 isolates ($n = 8$) of clinical and veterinary origin were obtained from The Department of Medical Microbiology (Foresterhill, Aberdeen, UK), The National Collection of Type Cultures (CPHLS, London, UK) and PHLS (Cherry Orchard Hospital, Dublin, Ireland) (Table 1). *Escherichia coli* O157 isolates ($n = 8$) were recovered from beef trimmings at the National Food Centre or obtained from The National Collection of Type Cultures (CPHLS, Colindale, UK). Cultures of *E. coli* O145, O103 and *E. coli* NCTC 12100 were obtained from the National Collection of Type Cultures (CPHLS, Colindale, UK). All strains were stored on protect beads at -20°C according to manufacturer's instructions (Technical Services Consultants Ltd, Heywood, UK).

Cultures were confirmed as *E. coli* by growth on eosin methylene blue (EMB) agar and testing for the formation of indole (Oxoid, Basingstoke, UK), and as *E. coli* O26, *E. coli* O111 or *E. coli* O157 by latex agglutination (Denka Seiken, Tokyo, Japan) using the relevant O-somatic antigens and an antiserum test (Statens Serum Institut, Copenhagen, Denmark).

Isolates were examined for the presence of verotoxin (*vt*) genes by PCR amplification of the *vt1* and *vt2* genes using primers vt1F and vt1R to amplify a 180-bp amplicon of the A subunit coding region of *vt1* and primers vt2F and vt2R to amplify a 255-bp amplicon of the A subunit coding region of *vt2* (including *vt2* variants). PCR procedures were carried out using Taq Polymerase (Qiagen, Crawley, UK) following the manufacturers instructions and previously described procedures (Paton and Paton 1998) (Table 2). The presence of *vt* genes in all strains (Table 1) was also confirmed by independent PCR analysis by the CPHLS (Colindale, UK).

Meat samples

Retail minced beef was purchased from a range of butcher shops and held at 4°C for up to 24 h.

Table 1 *Escherichia coli* strains, genotypes and/or sources

Serogroup	Strain	Genotype		Source
		<i>vt1</i>	<i>vt2</i>	
<i>Escherichia coli</i> O26	M328	+	+	Clinical isolate
<i>Escherichia coli</i> O26	381	+	-	Unknown
<i>Escherichia coli</i> O26	332	+	-	Sick calves
<i>Escherichia coli</i> O26	WF006	+	-	Sick calves
<i>Escherichia coli</i> O26	H11	+	-	Sick calves
<i>Escherichia coli</i> O26	8783 K60(B6):H11	-	-	Clinical isolate
<i>Escherichia coli</i> O26	354	+	-	Human isolate
<i>Escherichia coli</i> O26	361	+	-	Clinical isolate
<i>Escherichia coli</i> O111	4892	-	-	University of Aberdeen
<i>Escherichia coli</i> O111	378 CRA12847 0001561	+	+	Campden and Chorleywood Food
<i>Escherichia coli</i> O111	359	+	-	Human isolate
<i>Escherichia coli</i> O111	8008 K58(B4):H2	-	-	Clinical isolate
<i>Escherichia coli</i> O111	8179 K58(B4):H2	-	-	Clinical isolate
<i>Escherichia coli</i> O111	DD5	+	+	SAC inverness
<i>Escherichia coli</i> O111	E1278	-	-	SAC inverness
<i>Escherichia coli</i> O111	9111	-	-	SAC Inverness
<i>Escherichia coli</i> O157	ENTC 9490	+	+	Food isolate
<i>Escherichia coli</i> O157	ATCC 43895	+	-	Food isolate
<i>Escherichia coli</i> O157	380-94	+	+	Food isolate
<i>Escherichia coli</i> O157	2	+	+	Food isolate
<i>Escherichia coli</i> O157	10	-	+	Food isolate
<i>Escherichia coli</i> O157	11	-	+	Food isolate
<i>Escherichia coli</i> O157	13	-	+	Food isolate
<i>Escherichia coli</i> O157	57	-	+	Food isolate
<i>Escherichia coli</i> O145	NC 10279	-	-	
<i>Escherichia coli</i> O103	NC 9103	-	-	
<i>Escherichia coli</i>	NCTC 12100	-	-	

Table 2 Primers and probes used in detection of VT or serotype-specific genes (O157, O26 and O111)

Primer/probe	Organism	Tm (°C)	Target gene	Sequence	Position from start of <i>Escherichia coli</i> gene
VT1F	VTEC	55.4	Vt1	ATAAATCGCCATTCGTTGACTAC	454-476
VT1R	VTEC	57.0	<i>Vt1</i>	AGAACGCCCACTGAGATCATC	633-613
VT1 FL	VTEC	63.5	<i>Vt1</i>	CGTAACATCGCTCTTGCCACAGAC	545-522
VT1 LC	VTEC	63.0	<i>Vt1</i>	CGTCAGTGAAGTTCCACTATGCGA	519-496
VT2 S	VTEC	55.3	<i>Vt1</i>	GGCACTGTCTGAACTGCTC	606-625
VT2 R	VTEC	55.4	<i>Vt2</i>	TCGCCAGTTATCTGACATTCTG	860-839
VT2 FL	VTEC	62.4	Vt2	CCCCGAWACTCCGGAAGCAC	707-688
VT2 LC	VTEC	63.7	<i>Vt2</i>	TTGCTGATTCKCCCCAGTTCAGW	686-663
O157F	<i>E. coli</i> O157	56.9	<i>per</i>	TCTGCGCTGCTATAGGATTAGC	701-722
O157A	<i>E. coli</i> O157	56.0	<i>per</i>	CTTGTTTTCGATGAGTTTATCTGCA	926-903
O111S	<i>E. coli</i> O111	55.5	<i>wzy</i>	CTTTTTTTGAACCTACAGCAAGTAA	632-656
O111R	<i>E. coli</i> O111	54.5	<i>wzy</i>	GATAAACCAATGCTCCTATCACAC	859-836
O26FlipCF	<i>E. coli</i> O26	61.8	<i>FliC</i>	GCAGCGGATGGCAATGGGAAT	
O26 FliAR	<i>E. coli</i> O26	65.3	<i>FliA</i>	TCCACGCTCGCGGGCAGTC	120-102

Preparation of inocula

Individual protect beads containing VTEC isolates, i.e. *E. coli* O157, O111 or O26, and nonpathogenic *E. coli* (NCTC

12100), *E. coli* O103 or *E. coli* O145 where separately incubated statically in 10 ml of brain-heart infusion (BHI) (Oxoid) at 37°C overnight. One millilitre aliquots of the resultant cultures were transferred into fresh 10 ml of BHI

and further incubated at 37°C overnight. This process yielded *E. coli* cultures containing *ca* 10⁹–10¹⁰ CFU ml⁻¹.

Enrichment broths

Previous work carried out in our laboratory (Catarama *et al.* 2003) showed that a initial inoculum of *ca* 10 CFU g⁻¹ could be detected after a selective enrichment for 6 h using biochemical tests (indole test, latex and antiserum agglutination) therefore this initial inoculum was used again for detection using real-time PCR. The enrichment broths used for *E. coli* O26 and O111 have already been evaluated (Catarama *et al.* 2003). The enrichment broth used for *E. coli* O157 is an established standard method (ISO 16654).

Escherichia coli O26 (*n* = 8), O111 (*n* = 8) and O157 (*n* = 8) cultures and nonpathogenic *E. coli* (NCTC 12100), *E. coli* O103 or *E. coli* O145 were serially diluted in maximum recovery diluent (Oxoid), to obtain inocula containing *ca* 10 CFU g⁻¹, inoculated into 25 g samples of minced beef samples, and suspended in broth media as follows:

- i *Escherichia coli* O26 in 225 ml of tryptone soya broth (TSB) (Oxoid) supplemented with vancomycin (Sigma, Poole, UK) at 40 mg l⁻¹, cefixime (Fujisawa, Osaka, Japan) at 50 µg l⁻¹ and potassium tellurite (Sigma) at 2.5 mg l⁻¹ (TSB + CVPt).
- ii *Escherichia coli* O111 in 225 ml of TSB supplemented with vancomycin (Sigma) at 40mg l⁻¹ and cefixime (Fujisawa) at 50 µg l⁻¹ (TSB + CV).
- iii *Escherichia coli* O157 in 225 ml of *E. coli* (EC) broth with novobiocin 20 mg l⁻¹ (Sigma) (EC + N).

Minced beef samples containing nonpathogenic serotypes, i.e. *E. coli* (NCTC 12100), *E. coli* O103 or *E. coli* O145, and uninoculated minced beef samples were suspended at *ca* 10 CFU g⁻¹ in 225 ml of all of the above broth media.

All suspensions were stomached (Stomacher 400 Circulator; Seward, London, UK) at 230 rev min⁻¹ for 2 min and incubated statically at 41.5°C for 6 h (Catarama *et al.* 2003).

The above inoculum preparation, inoculation and enrichment procedures were repeated on three occasions following enrichment at 41.5°C for 6 h. The numbers of *E. coli* inoculated into meat were confirmed by plating onto MacConkey agar and counting after incubation at 37°C for 18 h.

Extraction methods

Direct extraction from enrichment broths. After enrichment each broth was shaken gently to evenly disperse the content, and a 25-ml aliquot was removed. Each aliquot was centrifuged at 100 *g* for 5 min, to separate fat particles and precipitate larger tissue debris. A 1-ml sample of the

central aqueous layer was transferred into a 1.5-ml microcentrifuge tube and centrifuged at 5000 *g* min⁻¹ for 10 min (Ge *et al.* 2002) The resultant supernatant was discarded and the cell pellet processed to recover bacterial DNA.

Extraction using immunomagnetic separation. Immunomagnetic beads (Dynal, Oslo, Norway) coated with antibodies selective for the O-antigen of *E. coli* O157, O26 or O111 were used according to manufacturers instructions. Briefly, 1 ml aliquots of the enriched culture was incubated in 1.5 ml microcentrifuge tubes with 25 µl of the immunomagnetic separation (IMS) bead suspension for 10 min at room temperature. The immunomagnetic beads were then recovered from the mixture using a magnetic separator MPC-M (Dynal) and washed twice with 1 ml 0.01 mol l⁻¹ phosphate buffer solution, pH 7.2 (ISO Method 1998). The resultant supernatant was discarded and the cell-bead complex was processed to recover bacterial DNA.

DNA extraction. DNA was extracted from the bacterial pellets isolated from the direct centrifugation extraction method and the cell-bead IMS complex using the DNAeasy Tissue Kit (Qiagen). DNA was extracted from the centrifuged bacterial pellet according to DNAeasy Tissue Kit protocol for the extraction of DNA from Gram-negative bacteria and eluted in sterile water (Sigma) and immediately analysed by PCR, or frozen at -20°C for later use. The IMS cell-bead complex was suspended in 180 µl buffer ATL (DNAeasy Tissue Kit; Qiagen) and the DNA was also isolated from the cells according to DNAeasy Tissue Kit protocol for the extraction of DNA from Gram-negative bacteria. The recovered DNA was eluted in sterile water and used immediately in real-time PCR assays or stored at -20°C for later use.

Primer and probe design

PCR primers were designed to amplify and detect the *vt1* gene (VTEC-specific), *vt2* gene (VTEC-specific), *per* gene of *rfb* cluster (O157-specific) or *mzy* gene of *rfb* cluster (O111-specific) genes using sequencing data available in the EMBL databank. Approximately 700/958 bp of the *vt1* gene of *E. coli* O26 M328 (accession no. AJ537519), O26 381 (accession no. AJ537520) and O111 378 (accession no. AJ537515), and *ca* 760/960 bp of the *vt2* genes of O26 M328 (accession no. AJ543441) and O111 378 (accession no. AJ543442) were sequenced. Pair-wise alignments with available O157 *vt1* (accession no. L04539) and O111 *vt2* (accession no. L11078) sequences were produced using multiple sequence alignment software available on the internet (<http://www.toulouse>).

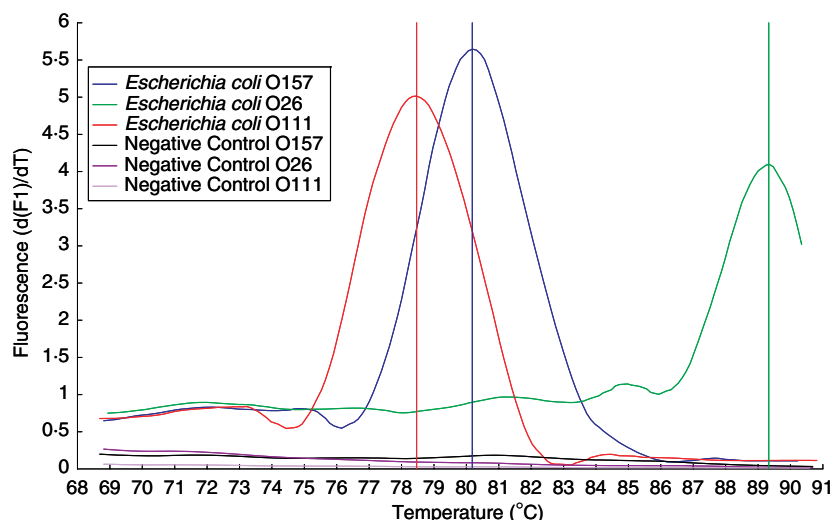


Fig. 1 T_m^* curve analysis of SYBR green 1 species-specific assay using real-time PCR. * T_m was 80.2°C for *Escherichia coli* O157, 78.3°C for *E. coli* O111 and 89.2°C for *E. coli* O26

inra.fr/multalin.html), in order to check for strain-specific variations in the genes. Sequence data for the O111 *wzy* gene putative O antigen polymerase in the *rfb* cluster (accession no. AF078736) was used to design species-specific primers (O111S, O111R), whilst sequence data for the O157 *per* gene perosamine synthetase in the *rfb* cluster (accession no. AF061251) was used to design sequence-specific primers (O157F, O157A) (Table 2).

Each set of PCR probes was designed to detect the *vt1* and *vt2* genes. The multiple sequence alignments outlined above were used to design sequence-specific probes for *vt1* (VT1FL, VT1LC) and *vt2* (VT2FL, VT2LC) (Table 2). Each probe was labelled, one with fluorescein (the donor dye) at the 3'-end and the other one with LightCycler-Red 640 (the acceptor dye) at the 5'-end. Primers and probes were designed and made by Tib-MolBiol (Berlin, Germany).

Primer and probe specificity

The specificity of the serogroup-specific primers and the *vt1*, *vt2* primers and hybridization probes were evaluated by real-time PCR using DNA isolated from pure cultures of all the strains in Table 1. Preliminary studies (results not shown) confirmed that the primers and probes did not amplify DNA from common meat microflora, e.g. *Pseudomonas*, *Listeria*, *Salmonella* and *Yersinia*.

Inverse PCR

As no serogroup-specific sequence data was available for *E. coli* O26, it was necessary to generate sequence data by inverse PCR using the O26 *fliC* gene sequence available in the EMBL databank (accession no. AJ243796). Briefly, 2 µg of O26 DNA was digested with 4 U EcoRI (New England Biolabs, Beverly, MA, USA) at 37°C for 6 h. After heat

inactivation of the restriction enzyme at 68°C for 15 min, the DNA fragments were diluted in water to 25 ng l⁻¹ and incubated with T4 DNA ligase (15 U l⁻¹) (New England Biolabs) at 16°C for 18 h to circularize the DNA. Ten-nanogram amounts of circularized template DNA were used in a PCR mix with ca 0.5 µmol l⁻¹ of each primer (flipCR – CGGAAAGTGTGGCAGCCTTGT and InvPCRF – TTCTGCCCGTAGCCGTATCGA), 2.5 U *Taq* DNA Polymerase, 200 µmol l⁻¹ of each of dNTP and 1X PCR buffer (containing 1.5 nmol l⁻¹ MgCl₂) and H₂O to a final volume of 50 µl. Thirty-five polymerization cycles were performed under the following conditions: initial denaturation at 94°C for 1 min, primer annealing at 44°C for 1 min, extension at 72°C for 3 min. PCR products were separated by gel electrophoresis and stained with ethidium bromide to detect an amplicon of 215 bp (PCR-1). This amplicon was sequenced commercially by MWG Biotech AG (Ebersberg, Germany). The PCR experiment was repeated using an alternate restriction enzyme (4 U of Asp 718) and alternate primers (Inv2R – AAGTGTAATTAACGCAGCA and Inv2f – ACTCATATAACGCAGGGCTG) designed from PCR-1 fragment. An amplicon of 133 bp was generated (PCR-2). A unique region of 348 bp downstream of *fliC* encompassing an intergenic region and the partial sequence of the *fliA* gene of *E. coli* O26 M328 strain (accession no. AJ537491) was obtained (Fig. 1). The real-time PCR primer flipCF was designed in the *fliC* gene and fliAR was designed in the *fliA* gene to amplify this *E. coli* O26-specific sequence (Table 2).

Real-time PCR

Amplification and detection were carried out in a LightCycler Instrument (Roche, Germany). The Faststart SYBR Green I master mix kit (Roche, Lewes, UK) was used in all

Programme	Target temperature	Hold time (s)	Slope (°C s ⁻¹)	Acquisition mode
PCR syber green protocol				
Denaturation (one cycle)	95	600	20	None
Amplification (35 cycles)	95	15	20	None
	54*	5	20	None
	72	12*	20	Single
Melting curve analysis (one cycle)	95	0	20	None
	65	15	20	None
	95	0	0.1	Continuous
Cooling (one cycle)	40	30	20	None
Hybridization probe analysis				
Denaturation (one cycle)	95	30	20	None
Amplification (35 cycles)	95	0	20	None
	50	15	20	Single
	72	13	20	None
Cooling (one cycle)	40	30	20	None

Table 3 Real-time PCR conditions for amplification of *vt1*, *vt2* or serotype-specific genes (O157, O111 and O26)

*O26-specific PCR amplification programme: target temperature 60°C and hold time 40 s.

experiments targeting species-specific reactions. The Hybridisation Probes master mix kit (Roche, UK) was used in experiments targeting *vt1* and *vt2* genes. Optimization of real-time PCR procedures included titration of a range of MgCl₂ and primer concentrations, and optimization of annealing temperatures and extension times. The optimized conditions used in the analyses are outlined in Table 3. Real-time PCR using Faststart SYBR Green was carried out using 3 µl of extracted DNA, 0.8 µl MgCl₂ (final concentration 1 mmol l⁻¹), 2 µl of 10X LightCycler DNA Master SYBR Green I and 2 µl of each primer (final concentration 0.5 µmol l⁻¹) made up to a total volume of 20 µl in sterile water. Real-time PCR using Faststart Hybridisation Probe was carried out using 3 µl of extracted DNA, 1 µl MgCl₂ (final concentration 1.25 mmol l⁻¹), 10 µl of 10X LightCycler DNA Master SYBR Green I, 2 µl of each primer (final concentration 0.5 µmol l⁻¹) and 1 µl of each hybridization probes (final concentration 0.15 µmol l⁻¹) made up to a total volume of 20 µl in sterile water.

All light cycler analysis was carried out using the second derivative maximum option of the light cycler software (Version 3.01). The 'Fit Point Method' was used in the light cycler software at which the crossing point (CP) was measured at constant fluorescence level. Real-time PCR efficiencies were calculated from the given slopes in light cycler software. The corresponding real-time PCR efficiency (*E*) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{(-1/\text{slope})}$.

Quantification of VTEC DNA

Production of standard curves. Pure culture strains of *E. coli* O26, O157 and O111 were grown in BHI (Oxoid)

overnight. DNA was isolated from each pure culture using the DNAeasy Tissue Kit protocol for the extraction of DNA from Gram-negative bacteria (Qiagen). The amounts of DNA recovered were estimated using spectrophotometric mean (Specgene, Techne, UK) and the LightCycler quantification software was used to generate standard curves calibrating the amount of fluorescence emitted during real-time PCR with known DNA concentrations (Fig. 3). This procedure is outlined in detail in the Roche LightCycler Manual Version 3.5. CP cycles vs total DNA concentration input were plotted to calculate the slope (mean ± S.D.).

PCR primers O157F and O157A were used on *E. coli* O157 DNA, primers O111S and O111R was used on *E. coli* O111 DNA, and primers O26FlipCF and O26FliAR were used on *E. coli* O26 DNA (Fig. 2).

Determination of the ratio of VTEC DNA to meat spoilage bacteria DNA in PCR products

The ratio of *E. coli* O157, O111 or O26 to overall meat spoilage bacterial DNA in the reaction was determined using

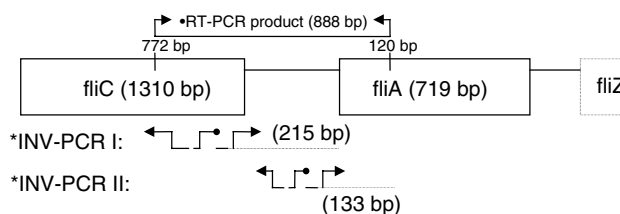


Fig. 2 Map of *fliC-fliA* gene region of *Escherichia coli* O26 H-antigen region. *Inverse PCR maps (PCR I and II) showing newly sequenced partial sequence. • Real-time PCR primers and 888 bp amplicon for detection of *E. coli* O26-specific sequence

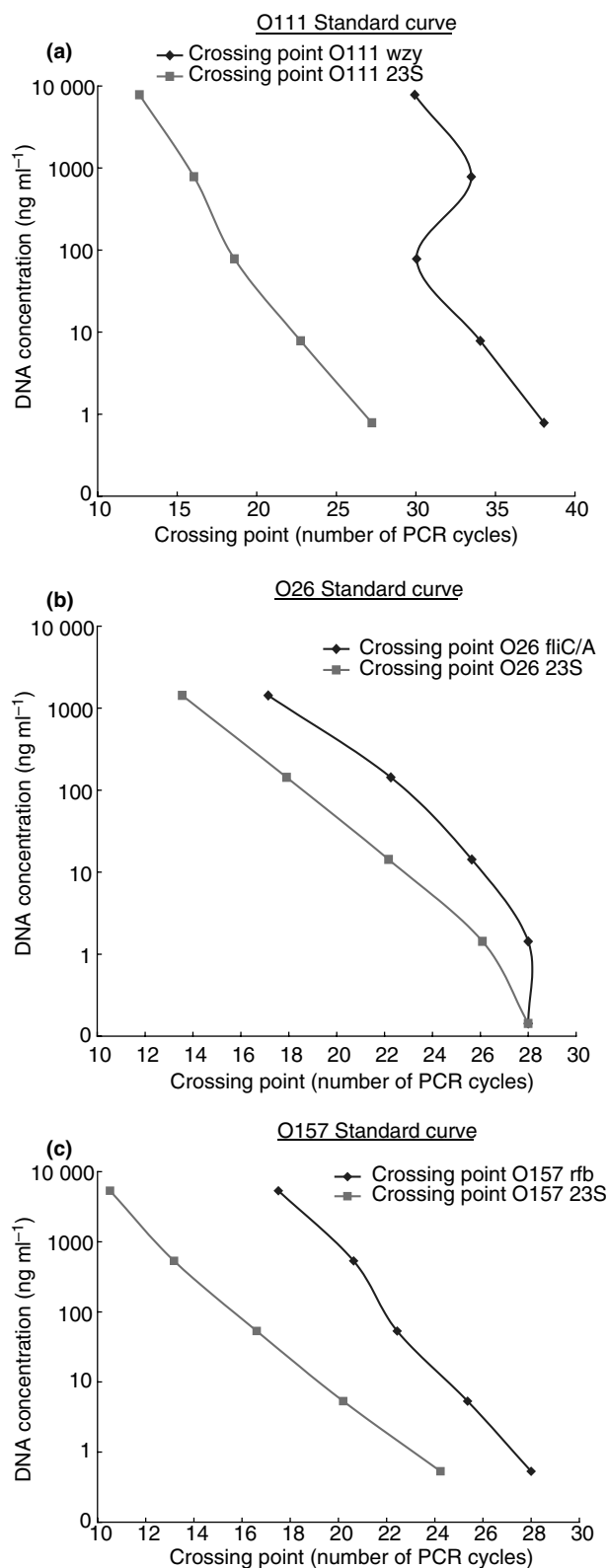


Fig. 3 Standard curves to quantify *Escherichia coli* O111, O157 and O26-specific DNA and background DNA

the LightCycler relative quantification software (version 1.01; Roche Molecular Biochemicals), to compare the estimated amounts of specific serogroup (O26, O157 and O111) target PCR product with the estimated amounts of a reference (nontarget) product after the PCR process using primers PF (AAGCTTGCTGGAGGTATCAGAAGTGC) and PR (CTCCGCCCTCCATCGCAGT) (Fig. 3). This reference product was derived from the 23S rRNA sequence of eight common meat spoilage organisms (*Pseudomonas fluorescens*, *P. putida*, *P. fragi*, *P. auerofaciens*, *Acinetobacter calcoaceticus*, *Enterobacter liquifaciens*, *Flavobacterium* spp. *Moraxella* spp. and *Brochothrix thermosphactum*) which is found at a constant copy number in most common meat pathogens (Venkitanarayanan *et al.* 1996). Preliminary studies (results not shown) confirmed that the PF and PR primers did not amplify DNA from beef meat.

PCR quantification analysis. Two separate PCR calibrators of known DNA concentration were included in each real-time PCR run to amplify the target gene and the reference gene. These were necessary to normalize the final results. In each analysis, the target/reference ratio of all the samples was divided by the target/reference ratio of the calibrator. The ratio of target DNA to reference DNA was also compared using two DNA extraction methods (IMS and centrifugation) (Table 5).

Equation 1 shows the equation for the relative expression ratio derived in this study. The ratio of target gene is expressed in a sample vs a control in comparison with a reference gene.

$$\text{Ratio} = \left\{ \begin{array}{l} \text{Median (target)/Median (reference)} \\ \times \text{Multiplication factor.} \\ [\text{Median (target}_{\text{cal}})/\text{Median (reference}_{\text{cal}})] \\ \times \text{Correction factor.} \end{array} \right.$$

All the enrichment and real-time PCR detection experiments outlined above were carried out in triplicate on different days using a freshly prepared inocula, meat samples and enrichment broths.

RESULTS

Optimization of real-time PCR and primer and probe specificity

The optimized components and conditions of the PCR reaction (MgCl_2 concentration, primer concentration, annealing temperature and time) are presented in Table 3. The specificity of the *vt1* and *vt2* hybridization primers and probes against DNA from a range of strains of VTEC was demonstrated by successful PCR amplification and subsequent

detection of target fragments. Amplification signals were produced for those strains that harboured the target genes. There were no false positives, i.e. no amplification signals were produced for those strains which lacked these target gene (Table 4). In general, the results obtained with primer and probes targeting *vt1* and *vt2* genes were identical to those obtained by PCR using primers *vt1F/vt1R* and *vt2F/vt2R* on pure culture DNA and to results obtained from an independent PCR analysis for the presence of *vt* genes by the CPHLS (Table 4). However, two false-negatives were obtained using *E. coli* O157 ATCC 43895 and *E. coli* O157 strain 2 (Table 4). The melting curve analysis of the amplicons from the O157, O26 and O111-specific PCR, and the expected temperature of melting for each amplicon are shown in Fig. 1.

Quantification of VTEC DNA: direct extraction and IMS extraction

There were no notable differences between VTEC DNA yield using the direct extraction method and DNA yield using the IMS extraction method (Table 5).

Quantification of VTEC DNA to DNA of background flora

The ratio of VTEC DNA to DNA from other bacteria and the recoveries by IMS and direct extraction are presented in Table 5.

Escherichia coli O26

Table 5 presents the relative ratios of concentration of target gene (VTEC species-specific) to reference gene (meat microflora) recovered from all the *E. coli* O26 strains by IMS and direct DNA extraction methods. The ratio of target DNA to reference DNA was higher from DNA extracted by IMS than direct extraction in seven of the eight *E. coli* O26 strains (Table 5).

Escherichia coli O111

Table 5 presents the relative ratios of concentration of target gene (VTEC species-specific) to reference gene (meat

Table 4 Detection of target gene sequences in VTEC spiked samples

Serogroup	Strain	CFU g ⁻¹ inoculum	Genotype		Detection by <i>vt</i> primers/ probes		Detection with O26-specific primers	Detection with O157-specific primers	Detection with O111-specific primers
			<i>vt1</i>	<i>vt2</i>	<i>vt1</i>	<i>vt2</i>			
<i>Escherichia coli</i> O26	M328	4	+	+	+	+	+	-	-
<i>Escherichia coli</i> O26	381	8	+	-	+	-	+	-	-
<i>Escherichia coli</i> O26	332	16	+	-	+	-	+	-	-
<i>Escherichia coli</i> O26	WF006	8	+	-	+	-	+	-	-
<i>Escherichia coli</i> O26	H11	6	+	-	+	-	+	-	-
<i>Escherichia coli</i> O26	8783	6	-	-	-	-	+	-	-
<i>Escherichia coli</i> O26	354	10	+	-	+	-	+	-	-
<i>Escherichia coli</i> O26	361	16	+	-	+	-	+	-	-
<i>Escherichia coli</i> O111	4892	10	-	-	-	-	-	-	+
<i>Escherichia coli</i> O111	378	12	+	+	+	+	-	-	+
<i>Escherichia coli</i> O111	359	20	+	-	+	-	-	-	+
<i>Escherichia coli</i> O111	8008	6	-	-	-	-	-	-	+
<i>Escherichia coli</i> O111	8179	12	-	-	-	-	-	-	+
<i>Escherichia coli</i> O111	DD5	14	+	+	+	+	-	-	+
<i>Escherichia coli</i> O111	E1278	12	-	-	-	-	-	-	+
<i>Escherichia coli</i> O111	9111	1	-	-	-	-	-	-	+
<i>Escherichia coli</i> O157	ENTC9490	12	+	+	+	+	-	+	-
<i>Escherichia coli</i> O157	ATCC43895	9	+	-	+	-	-	+ (-1 rep)	-
<i>Escherichia coli</i> O157	380-94	12	+	+	+	+	-	+	-
<i>Escherichia coli</i> O157	2	10	+	+	+	+	-	+ (-1 rep)	-
<i>Escherichia coli</i> O157	10	10	-	+	-	+	-	+	-
<i>Escherichia coli</i> O157	11	8	-	+	-	+	-	+	-
<i>Escherichia coli</i> O157	13	9	-	+	-	+	-	+	-
<i>Escherichia coli</i> O145	NC 10279	12	-	-	-	-	-	-	-
<i>Escherichia coli</i> O103	NC 9103	12	-	-	-	-	-	-	-
<i>Escherichia coli</i>	NCTC 12100	8	-	-	-	-	-	-	-

Table 5 Quantification and detection of IMS and directly extracted VTEC gene target real-time PCR

Bacterial strain/serotype	IMS/direct extraction	PCR detection (target gene)	DNA concentration from standard curve	Crossing point target	Crossing point reference	Ratio concentration target/reference
<i>Escherichia coli</i> O26 Irish	IMS	√	0.30	25.98	20.83	2.6×10^{-1}
	Direct	√	2.86	22.73	15.00	5.0×10^{-2}
<i>Escherichia coli</i> O26 381	IMS	√	3.93	22.21	16.81	1.8×10^{-1}
	Direct	√	0.69	25.04	13.00	1.0×10^{-2}
<i>Escherichia coli</i> O26 332	IMS	√	6.84	21.30	16.66	2.6×10^{-1}
	Direct	√	0.54	25.45	15.00	1.0×10^{-2}
<i>Escherichia coli</i> O26 WF006	IMS	√	1.45	22.99	17.68	2.9×10^{-1}
	Direct	√	0.11	27.24	13.21	3.3×10^{-3}
<i>Escherichia coli</i> O26 H11	IMS	√	1.82	22.62	17.78	3.7×10^{-1}
	Direct	√	0.00	37.73	13.84	2.9×10^{-5}
<i>Escherichia coli</i> O26 8783	IMS	√	3.76	21.44	17.17	4.7×10^{-1}
	Direct	√	10.00	19.84	13.85	1.7×10^{-1}
<i>Escherichia coli</i> O26 354	IMS	√	0.96	24.51	14.63	2.0×10^{-2}
	Direct	√	3.41	22.44	18.18	3.4×10^{-1}
<i>Escherichia coli</i> O26 361	IMS	√	9.06	20.84	16.43	2.9×10^{-1}
	Direct	√	7.40	21.17	14.54	9.0×10^{-2}
<i>Escherichia coli</i> O111 4892	IMS	√	189.6	23.28	15.93	38.6
	Direct	√	498.0	21.74	11.43	5.45
<i>Escherichia coli</i> O111 378	IMS	√	54.50	25.27	18.76	74.7
	Direct	√	55.32	25.24	14.92	6.86
<i>Escherichia coli</i> O111 359	IMS	√	189.6	26.61	17.20	13
	Direct	√	498.0	25.09	12.54	2
<i>Escherichia coli</i> O111 8008	IMS	√	189.6	23.28	17.52	104
	Direct	√	137.3	23.80	14.49	11.7
<i>Escherichia coli</i> O111 8179	IMS	√	0	28.82	24.91	Not quantifiable
	Direct	√	0	38.87	14.79	Not quantifiable
<i>Escherichia coli</i> O111 DD5	IMS	√	80.22	24.65	19.06	1.27
	Direct	√	79.94	24.66	15.02	1.0×10^{-1}
<i>Escherichia coli</i> O111 E1278	IMS	√	45.29	25.56	18.49	5.0×10^{-1}
	Direct	√	48.29	25.46	15.31	8.0×10^{-2}
<i>Escherichia coli</i> O111 9111	IMS	√	0.45	32.90	25.03	5.4×10^{-1}
	Direct	√	0.49	32.77	18.02	7.0×10^{-2}
<i>Escherichia coli</i> O157 9490	IMS	√	0	30.85	23.69	2.0×10^{-2}
	Direct	√	0	29.25	15.97	1.0×10^{-2}
<i>Escherichia coli</i> O157 43895	IMS	X	0	38.92	26.72	2.10×10^{-5}
	Direct	√	0	32.17	17.96	2.34×10^{-3}
<i>Escherichia coli</i> O157 380-94	IMS	√	0	28.60	20.60	8.0×10^{-2}
	Direct	√	0	28.19	29.07	$3. \times 10^{-2}$
<i>Escherichia coli</i> O157 2	IMS	√	0	32.30	23.94	1.0×10^{-2}
	Direct	X	0	33.68	16.19	3.15×10^{-4}
<i>Escherichia coli</i> O157 10	IMS	√	0	29.54	21.14	4.0×10^{-2}
	Direct	√	0	30.61	19.08	1.0×10^{-2}
<i>Escherichia coli</i> O157 11	IMS	√	0	30.35	23.28	4.74×10^{-2}
	Direct	√	0	31.71	18.03	6.29×10^{-3}
<i>Escherichia coli</i> O157 13	IMS	√	0	35.52	24.87	8.01×10^{-4}
	Direct	√	0	33.75	18.58	1.65×10^{-3}
<i>Escherichia coli</i> O145		X				
<i>Escherichia coli</i> O103		X				
<i>Escherichia coli</i> NCTC 12100		X				

microflora) recovered from all the *E. coli* O111 strains by IMS and direct DNA extraction methods. The ratio of target DNA to reference DNA was highest from DNA extracted by IMS than direct extraction in all experiments using *E. coli* O111 strains. However, one O111 strain (8179) had such a low DNA concentration that the DNA could not be quantified using the O111-specific standard curve. (Table 5).

Escherichia coli O157

The relative ratio concentration for all the *E. coli* O157 strains, i.e. ratio of target to reference gene for IMS and direct extraction was very low. However, *E. coli* O157 was present as it was detected by PCR and temperature of melting analysis in six O157 strains extracted using the IMS step and six strains extracted directly from the enriched broth (Table 5).

DISCUSSION

Under the enrichment conditions and procedures used in this study, all examined strains of *E. coli* O26 and *E. coli* O111 grew to cell concentrations that could be consistently detected by subsequent real-time PCR analysis. Identical amplicons were detected by both DNA extraction/recovery methods. These results are similar to the findings by Catarama *et al.* 2003, where *E. coli* O26 and O111 inoculated at the same level (10 CFU g⁻¹) were identified by serological and biochemical examinations after enrichment in identical conditions. This low initial inoculum was similar to findings by Sharma (2002), who reported the detection, by real-time PCR, of *E. coli* O26, O111 and O157 using *eae*-specific probes at a level of 15, 1.5 and 1.5 CFU g⁻¹ of beef, respectively, whilst the detection sensitivity of the vt1 and vt2 probes in beef was 1.2 CFU g⁻¹. However, it is different from the findings by Sharma (2002), in that the enrichment time for the detection of *E. coli* O26 and O111 in this study was only 6 h compared with 16 h in the Sharma study. No previous work has been reported on the identification of serogroup-specific *fliA/fliC* genes of *E. coli* O26 using molecular techniques. Therefore, this is the first reported method for the identification of *E. coli* O26 serogroup-specific *fliA/fliC* genes using real-time PCR. The specific implications of this is that *E. coli* O26, and O111 can be identified faster than previously published methods.

In the case of *E. coli* O157, less consistent results were obtained, in that only six of seven serogroups gave positive results following IMS or direct DNA extraction. This may be due to the fact that smaller concentrations of DNA were obtained from O157 strains, irrespective of the DNA extraction method employed. These smaller yields may be

related to relatively slower growth of *E. coli* O157:H7 under the conditions used. Such slower growth rates would be of increased significance in this procedure, using a much shorter (6 h) enrichment period, in comparison with the 24 h period recommended within the ISO 16654 method. While a shorter enrichment method may be preferable, a longer enrichment period, as recommended in the ISO method, is likely to avoid the increased risks of false-negative results for slower growing serogroups. Further work may be necessary to identify the optimum trade off point between speed and accuracy in PCR examination of O157 serogroups.

This study did not observe any notable qualitative and quantitative differences between the PCR patterns obtained from DNA samples recovered by IMS, and by direct extraction for *E. coli* O26 and O111. This suggests that no advantage is to be gained by the more complex, slower and expensive IMS recovery method. *Escherichia coli* O157 was observed both qualitatively (lower fluorescence signal) and quantitatively at a much lower level using both IMS and direct extraction methods. However, as the *E. coli* O157 DNA concentration present from both extraction methods was lower than O26 and O111, we assume it is due to the relatively slower growth of *E. coli* O157:H7 under the conditions used.

This study has developed and optimized a real-time PCR assay, which can rapidly detect organisms harbouring the verotoxin genes, and perhaps equally significantly, can specifically identify positive samples as being derived from one of the three (O26, O111 or O157) serogroups most commonly responsible for haemorrhagic colitis and HUS (Wells *et al.* 1983; Johnson *et al.* 1996). Although a small number of conventional PCR-based assays have been described for the detection of O26, O111 or O157 serogroups (Louie *et al.* 1998; Paton and Paton 1998), these procedures are not suitable for quantification of target organisms.

Future possibilities for this work is to successfully apply a rapid (24 h) and quantitative real-time PCR assay capable of detecting ca 10 CFU g⁻¹ VTEC in minced beef samples and in estimating initial VTEC DNA concentration and the ratio of VTEC to background meat pathogens within such complex food matrices. These rapid and sensitive detection methods could be used in routine food testing, especially in circumstances when 'evidence of the absence' of the pathogen is necessary.

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